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Evaluation of *p53* Alterations in Occult Lymph Node Metastases

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Background and Objectives: This study was designed to evaluate *p53* alterations in occult lymph node metastases.
Methods: We examined 41 patients with stage I non-small-cell lung cancer. We investigated *p53* gene mutation by polymerase chain reaction and single-strand conformation polymorphism analysis of exons 5–8, p53 protein accumulation by immunostaining with monoclonal antibody DO-7, and detection of tumor cells in lymph nodes by immunohistochemistry with monoclonal antibodies to cytokeratin (CK).
Results: *p53* gene mutation was detected in 34% of tumors and nuclear p53 accumulation in 46%. CK-positive cells in the hilar and mediastinal region lymph nodes were detected in 43.9% of patients and 29.3%, respectively. Of the 14 cases with *p53* mutation and the 19 cases with p53 accumulation, 12 and 15 had micrometastases in the hilar or mediastinal lymph nodes, respectively. However, *p53* alterations were not significantly associated with occult lymph node metastases. In cases with occult lymph node metastases, the 5-year survival was 81.9% for the *p53* wild-type group and 45.8% for the *p53* mutation group.
Conclusions: *p53* alterations are not correlated with occult lymph node metastases, while *p53* gene mutation is considered to be an unfavorable prognostic marker in patients with occult lymph node metastases.
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KEY WORDS: ***p53* mutation; micrometastasis; lung cancer**

INTRODUCTION

p53 gene is one of the most frequently mutated tumor-suppressor genes in human tumors. It regulates the cell cycle negatively through transactivation of the *p21/Waf1* gene and, in some cases, induces apoptosis through transactivation of the *Bax* gene [1,2]. In non-small-cell lung cancer (NSCLC), *p53* mutation is found in about half of all tumors [3–10], and it is more frequent in squamous cell carcinoma than in adenocarcinoma [3,5,6,9,10]. We have shown that mutation of the *p53* gene is associated with poor prognosis in early-stage cases [11]. It remains undetermined, however, whether or not the presence of *p53* gene mutation in NSCLC has any prognostic value [5,9,10,12,13].

Occult micrometastases in the lymph nodes of patients with lung cancer have also been identified by other investigators using immunohistochemical techniques [14,15]. We have shown that occult micrometastases in the mediastinal lymph nodes, detected with monoclonal antibodies to cytokeratin (CK), can be used to predict an early relapse in patients with stage I NSCLC [16].

Overexpression of the p53 protein in primary tumors has also been shown to correlate with an increased incidence of these micrometastatic cells in the bone marrow

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[17]. *p53* alteration may induce occult micrometastases and be associated with poor prognosis in early-stage cases of lung cancer. In this study, we thus analyzed the relationship between *p53* mutation, accumulation, and occult micrometastases in the lymph nodes of patients with stage I NSCLC.

MATERIALS AND METHODS

Patients

We studied 41 patients with stage I NSCLC (21 T1N0M0, 20 T2N0M0) who underwent lobectomy combined with formal mediastinal and hilar node dissection at the Department of Surgery II, Faculty of Medicine, Kyushu University, between January 1991 and December 1992. Disease staging was based on the TNM classification of the Union Internationale Contre Cancer [18]. The patients consisted of 22 males and 19 females. The median age of patients was 66 years, with a range of 35–78. The median Brinkman smoking index, defined as cigarettes per day × years, was 720, with a range of 0–5,600. Twenty-nine patients had adenocarcinoma, 11 squamous cell carcinoma, and 1 large cell carcinoma. The median number of lymph nodes available for examination from each patient was 20, with a range of 8–45.

Detection of Point Mutations in *p53* Tumor-Suppressor Gene

From frozen and formalin-fixed, paraffin-embedded samples, high-molecular-weight DNA was isolated as described previously [19]. Four fragments of DNA, each encompassing exons 5–8 of *p53*, were amplified by polymerase chain reaction (PCR). The nucleotide sequences of the primers and the PCR conditions are available upon request. Point mutations in the *p53* gene were detected by single-strand conformation polymorphism (SSCP) [20]. The PCR-SSCP technique was applied as described previously [11].

Immunohistochemistry of p53 Accumulation

Accumulation of p53 protein was detected using the monoclonal antibody DO-7 (Novocastra, Newcastle-upon-Tyne, UK). The DO-7 monoclonal antibody recognizes both wild-type and mutant p53 epitopes in formalin-fixed, paraffin-embedded tissues. After having deparaffinized all sections, we used the microwave antigen retrieval technique for 20 min at 95°C. Possible background staining was also removed by applying normal rabbit serum, diluted 1:10, for 15 min at room temperature. A mouse DO-7 monoclonal antibody, diluted 1:100 and left overnight at 4°C, was applied; then biotinylated secondary antibody and streptavidin with mouse radish peroxidase (Nichirei, Tokyo, Japan) were applied for 20 and 15 min, respectively. Next, all slides were counterstained with methyl green. Carcinomas were considered to accumulate p53 protein if 10% or more of the malignant cells contained immunohistochemistry reaction products in the nucleus.

Lymph Node Micrometastases Detected by CK

Paraffin-embedded tissue sections were floated onto lysine-coated slides. We used a primary monoclonal antibody to CK (clone CAM5.2; Becton Dickinson Immunocytometry Systems, San Jose, CA), the indirect staining technique, and the streptavidin-biotin-peroxidase complex method. These techniques were applied as described previously [16].

Statistical Analysis

A comparison of the proportion was done using the χ^2 test. Survival was calculated from the date of operation to either death or the date of the last follow-up (censored). Survival was analyzed according to the Kaplan-Meier method, and differences in distribution were evaluated via the log-rank test [21,22]. P values of <0.05 were defined as statistically significant.

RESULTS

Frequency of *p53* Mutation

Of the 41 specimens examined, 14 (34%) had mutation of the *p53* gene: 3 were in exon 5, 2 in exon 6, 5 in exon 7, and 5 in exon 8 (one sample had a double mutation in exons 6 and 7). The relationship between *p53* mutation and clinicopathological parameters is summarized in Table I. The association between the smoking index and *p53* mutation approached but did not reach significance. The association between age, T factor, and *p53* mutation was significant.

Immunohistochemistry of p53 Accumulation

Of the 41 specimens examined, 19 (46%) had accumulation of p53 protein. The relationship between p53 accumulation and clinicopathological parameters is summarized in Table I. There was no significant association between p53 accumulation and the parameters.

Lymph Node Micrometastases Detected by CK

We examined a total of 889 lymph nodes from 41 patients with conventional stage I disease. CK-positive cells were identified in 23 (5.6%) of 409 lymph nodes from patients with T1N0M0 disease and in 62 (12.9%) of 480 lymph nodes from patients with T2N0M0 disease in whom metastases had not been detected by a routine examination of hematoxylin and eosin–stained slides. Of the 21 patients with T1 disease, CK-positive cells in the hilar region lymph nodes were detected in 11 (52.4%) and those in the mediastinal region lymph nodes in 3 (14.3%). Of the 20 patients with T2 disease, CK-positive cells in the hilar region lymph nodes were detected in 7 (35.0%) and those in the mediastinal region lymph nodes in 9 (45.0%). Initially, all 41 patients were stage I, and

TABLE I. Correlation between *p53* Alterations and Clinicopathological Features

	Total	*p53* mutation	p53 accumulation
	41	14 (34%)	19 (46%)
Sex			
Female	19	4 (21%)	9 (47%)
Male	22	10 (45%)	10 (45%)
		($P = 0.1004$)	($P = 0.9025$)
Age (years)			
<66	19	3 (16%)	7 (37%)
≥66	22	11 (50%)	12 (55%)
		($P = 0.0212$)	($P = 0.2570$)
BI			
<720	23	5 (22%)	10 (43%)
≥720	18	9 (50%)	9 (50%)
		($P = 0.0583$)	($P = 0.6777$)
Histological type			
Adenocarcinoma	29	8 (28%)	11 (38%)
Squamous cell carcinoma	11	5 (45%)	7 (64%)
Large cell carcinoma	1	1 (100%)	1 (100%)
		($P = 0.2113$)	($P = 0.1915$)
T factor			
T1	21	4 (19%)	7 (33%)
T2	20	10 (50%)	12 (60%)
		($P = 0.0367$)	($P = 0.0870$)
CK-N			
N0	11	2 (18%)	4 (36%)
N1	18	6 (33%)	8 (44)
N2	12	6 (50%)	7 (58%)
		($P = 0.2734$)	($P = 0.5598$)

BI, Brinkman smoking index; CK-N, N factor restaged by CK.

after detection of micrometastases, 11 patients remained stage I, 18 patients were reclassified as stage II, and 12 patients were reclassified as stage IIIA.

Association Between p53 Alterations, Lymph Node Micrometastases, and Prognosis

An overall concordance between accumulation of p53 protein and mutation of the gene (both positive or both negative) was found in 68.3% (28/41) of tumors. Of the tumors with positive nuclear staining, 52.6% (10/19) contained *p53* mutation. Of the tumors with mutation, 71.4% (10/14) showed p53 accumulation. The distribution of point mutations in the *p53* gene and the relation with p53 accumulation are summarized in Table II. Interestingly, there was a significant association between p53 accumulation and *p53* mutation in exon 7 or 8 ($P = 0.0085$).

Of the 14 cases with *p53* mutation, 12 had micrometastases in the hilar or mediastinal lymph nodes. The distribution of point mutations in the *p53* gene and the relation with the presence of micrometastases in the lymph nodes are summarized in Table III. Of the 19 cases with p53 accumulation, 15 had micrometastases in the hilar or mediastinal lymph nodes. No alterations were

TABLE II. Distribution of Point Mutations in the *p53* Gene and Its Relation with the Presence of p53 Accumulation

	Point mutations in *p53*				
	Wild-type	Exon 5	Exon 6	Exon 7	Exon 8
Accumulation of p53 protein					
Positive	9	1	1	5	4
Negative	18	2	1	0	1
Total	27	3	2	5	5

TABLE III. Distribution of Point Mutations in the *p53* Gene and Its Relation with the Presence of Micrometastases in Lymph Nodes

	Point mutation in *p53*				
	Wild-type	Exon 5	Exon 6	Exon 7	Exon 8
CK-N0	9	1	0	1	0
CK-N1	12	0	2	2	3
CK-N2	6	2	0	2	2
Total	27	3	2	5	5

associated with the incidence of lymph node micrometastases (Table I).

The median length of follow-up on all patients was 78 months, with a range of 7–98 months. Figure 1 shows the recurrence-free survival in cases with CK-positive cells in the hilar or mediastinal lymph nodes. The 5-year recurrence-free survival was 66.7% for the *p53* wild-type group and 46.9% for the *p53* mutation group. The difference between the 2 modalities was not significant ($P = 0.1833$). Figure 2 shows the survival in cases with CK-positive cells in the hilar or mediastinal lymph nodes. The 5-year-survival was 81.9% for the *p53* wild-type group and 45.8% for the *p53* mutation group. The difference between the 2 modalities was significant ($P = 0.0293$). Positive nuclear p53 accumulation was associated with neither recurrence-free survival nor survival in cases with CK-positive cells in the hilar or mediastinal lymph nodes (data not shown).

DISCUSSION

The *p53* gene regulates the cell cycle negatively through transactivation of the *p21/Waf1* gene and, in some cases, induces apoptosis through transactivation of the *Bax* gene [1,2]. Based on these findings, *p53* mutation appears to correlate with the development and progression of cancer. Atula and associates [23] described a significant correlation between tumor size, histological differentiation, and *p53* mutation found in patients with tongue cancer.

We analyzed the relationship between *p53* mutation, accumulation, and occult micrometastases in the lymph nodes of patients with stage I NSCLC. We found that the association between the smoking index and *p53* mutation approached but did not reach significance and that there

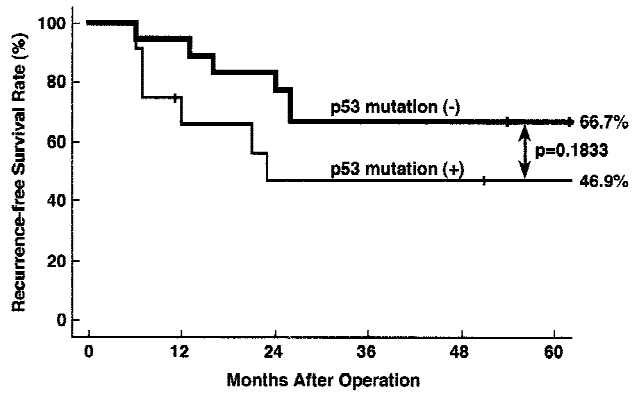

Fig. 1. Recurrence-free survival curves in cases with cytokeratin-positive cells in the hilar or mediastinal lymph nodes.

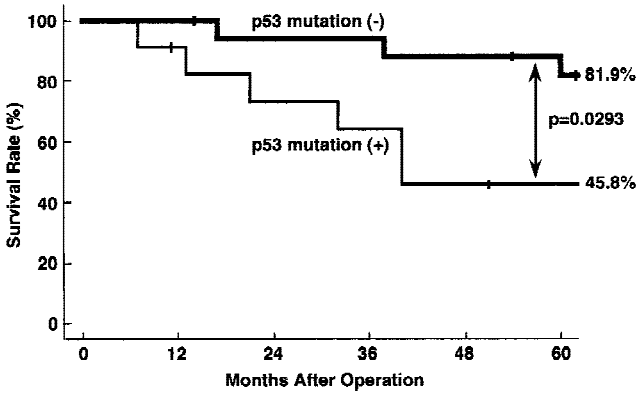

Fig. 2. Survival curves in cases with cytokeratin-positive cells in the hilar or mediastinal lymph nodes.

was a significant association between the age of patients, T factor, and *p53* mutation. The higher the age and the more the smoking index increased, the higher the incidence of *p53* mutation. In addition, *p53* mutation was associated with poor survival in cases with CK-positive cells in the hilar or mediastinal lymph nodes. However, there was no significant association between p53 accumulation and any of the parameters. Also, p53 accumulation was associated with neither recurrence-free survival nor survival in cases with CK-positive cells in the hilar or mediastinal lymph nodes. It may be due to these discrepancies between *p53* mutation and accumulation that the DO-7 monoclonal antibody recognized both wild-type and mutant p53 epitopes. Interestingly, there was a significant association between p53 accumulation and *p53* mutation in exon 7 or 8. It is suggested that the DO-7 antibody particularly recognized mutant p53 in exon 7 or 8. Neither *p53* mutation nor accumulation was associated with the incidence of lymph node micrometastases, which were few.

In our study, we analyzed only *p53* alteration; we did not analyze any correlation with other oncogenes. Metastatic progression was found to depend not only on *p53* mutations but also on other gene alterations. Taylor and associates [24] reported on a synergistic interaction between *ras*, *myc*, and mutant *p53* genes (proline-193 mutant form of *p53*) observed in focus formation and metastasis assays. They reported these results to support a working model of oncogene cooperativity in which alterations in *myc* and *p53* permit an elevated expression of *ras*, which is important as the mechanism affecting both cellular transformation in vitro and tumor dissemination in vivo. Metastatic progression depends on the increase in the malignant potential induced by multiple genetic changes.

The detachment of tumor cells is the first step in the process of metastasis and depends on the presence or absence of functional adhesion molecules. Passlick and associates [25] stated that a deficient or absent expression of intracellular adhesion molecule-1 might favor an early lymphatic spread of lung cancer cells. Sulzer and associates [26] showed a reduced expression of E-cadherin, an epithelial cell adhesion molecule, to be significantly correlated with increased lymphogenous metastasis and tumor dedifferentiation in NSCLC. When analyzing micrometastases in the lymph nodes, it thus appears important to look for not only alterations of oncogenes or tumor-suppressor genes but also the presence of adhesion molecules.

Of the 14 cases with *p53* mutation, 12 had micrometastases in the hilar or mediastinal lymph nodes. In the 5 cases in which we recognized mutations in exon 8 of the *p53* gene, micrometastases were detected. Huang and associates [27] reported mutation in exon 8 of *p53* to be a poor prognostic factor in patients with NSCLC. The poor prognosis in patients with mutation in exon 8 of *p53* might be associated with malignant behavior, such as the detachment of tumor cells and the lymphogenous spread of tumor.

In conclusion, *p53* mutation and accumulation are not correlated with micrometastases in the lymph nodes, while *p53* gene mutation is considered to be an unfavorable prognostic marker in cases with occult lymph node metastases of stage I NSCLC.

REFERENCES

1. El-Deiry WS, Tokino T, Velculesco VE, et al.: Waf1, a potential mediator of p53 tumor suppression. Cell 1993;75:817–825.
2. Selvakumaran M, Lin MS, Miyashita T, et al.: Immediate early up-regulation of *bax* expression by p53 but not TGFβ1: A paradigm for distinct apoptotic pathways. Oncogene 1994;9:1791–1798.
3. Iggo R, Gatter K, Bartek J, et al.: Increased expression of mutant forms of *p53* oncogene in primary lung cancer. Lancet 1990;335: 675–679.
4. Levine AJ, Momand J, Finlay CA: The *p53* tumour suppressor gene. Nature 1991;351:453–456.
5. McLaren R, Kuzu I, Dunnill M, et al.: The relationship of p53 immunostaining to survival in carcinoma of the lung. Br J Cancer 1992;66:735–738.

6. Miller CW, Simon K, Aslo A, et al.: *p53* mutations in human lung tumors. Cancer Res 1992;52:1695–1698.
7. Suzuki H, Takahashi T, Kuroishi T, et al.: *p53* mutations in non-small cell lung cancer in Japan: Association between mutations and smoking. Cancer Res 1992;52:734–736.
8. Mitsudomi T, Steinberg SM, Nau MM, et al.: *p53* gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of *ras* mutations and clinical features. Oncogene 1992;7:171–180.
9. Mitsudomi T, Oyama T, Kusano T, et al.: Mutations of the *p53* gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. J Natl Cancer Inst 1993;85:2018–2023.
10. Passlick B, Izbicki JR, Haussinger K, et al.: Immunohistochemical detection of p53 protein is not associated with a poor prognosis in non-small-cell lung cancer. J Thorac Cardiovasc Surg 1995;109: 1205–1211.
11. Fukuyama Y, Mitsudomi T, Sugio K, et al.: K-*ras* and *p53* mutations are an independent unfavourable prognostic indicator in patients with non-small-cell lung cancer. Br J Cancer 1997;75: 1125–1130.
12. Horio Y, Takahashi T, Kuroishi T, et al.: Prognostic significance of *p53* mutations and 3p deletions in primary resected non-small cell lung cancer. Cancer Res 1993;53:1–4.
13. Lee JS, Yoon A, Kalapurakal SK, et al.: Expression of p53 oncoprotein in non-small cell lung cancer: A favorable prognostic factor. J Clin Oncol 1995;13:1893–1903.
14. Chen ZL, Perez S, Holmes EC, et al.: Frequency and distribution of occult micrometastases in lymph nodes of patients with non-small-cell lung carcinoma. J Natl Cancer Inst 1993;85:493–498.
15. Passlick B, Izbicki JR, Kubuschok B, et al.: Immunohistochemical assessment of individual tumor cells in lymph nodes of patients with non-small-cell lung cancer. J Clin Oncol 1994;12:1827–1832.
16. Maruyama R, Sugio K, Mitsudomi T, et al.: Relationship between early recurrence and micrometastases in the lymph nodes of patients with stage I non-small-cell lung cancer. J Thorac Cardiovasc Surg 1997;114:535–543.
17. Ohgami A, Mitsudomi T, Sugio K, et al.: Micrometastatic tumor cells in the bone marrow of patients with non-small cell lung cancer. Ann Thorac Surg 1997;64:363–367.
18. Sobin LH, Wittekind C (eds): "TNM Classification of Malignant Tumours" (5th ed). New York: Wiley-Liss, 1997:93–97.
19. Sugio K, Kurata S, Sasaki M, et al.: Differential expression of *c-myc* gene and *c-fos* gene in premalignant and malignant tissues from patients with familial polyposis coli. Cancer Res 1988;48: 4855–4861.
20. Orita M, Iwahana H, Kanazawa H, et al.: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 1989;86: 2766–2770.
21. Kaplan E, Meier P: Non-parametric estimation from incomplete observation. J Am Stat Assoc 1958;53:457–481.
22. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemo Rep 1966;50: 163–170.
23. Atula S, Kurvinen K, Grénman R, et al.: SSCP pattern indicative for *p53* mutation is related to advanced stage and high-grade of tongue cancer. Oral Oncol Eur J Cancer 1996;32B:222–229.
24. Taylor WR, Egan SE, Mowat M, et al.: Evidence for synergistic interactions between ras, myc and a mutant form of p53 in cellular transformation and tumor dissemination. Oncogene 1992;7:1383–1390.
25. Passlick B, Pantel K, Kubuschok B, et al.: Expression of MHC molecules and ICAM-1 on non-small cell lung carcinoma: Association with early lymphatic spread of tumour cells. Eur J Cancer 1996;32:141–145.
26. Sulzer MA, Leers MPG, van Noord JA, et al.: Reduced E-cadherin expression is associated with increased lymph node metastasis and unfavorable prognosis in non-small cell lung cancer. Am J Respir Crit Care Med 1998;157:1319–1323.
27. Huang C, Taki T, Adachi M, et al.: Mutations in exon 7 and 8 of p53 as poor prognostic factors in patients with non-small cell lung cancer. Oncogene 1998;16:2469–2477.